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A quantitative analysis of objective feather colour assessment:

measurements in the lab are more reliable than in the field

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1   **Abstract**

2   The evolution of animal colouration is importantly driven by sexual selection operating on traits used to

3   transmit information to rivals and potential mates, which therefore, have major impacts on fitness.

4   Reflectance spectrometry has become a standard colour-measuring tool, especially after the discovery of

5   tetrachromacy in birds and their ability to detect UV. Birds' plumage patterns may be invisible to humans,

6   necessitating a reliable and objective way of assessing colouration not dependent on human vision.

7   Plumage colouration measurements can be taken directly on live birds in the field or in the lab (e.g. on

8   collected feathers). Therefore, it is essential to determine which sampling method yields more repeatable

9   and reliable measures, and which of the available quantitative approaches best assess the repeatability of

10   these measures. Using a spectrophotometer, we measured melanin-based colouration in barn swallows'

11   (*Hirundo rustica*) plumage. We assessed the repeatability of measures obtained with both traditional

12   sampling methods separately to quantitatively determine their reliability. We used the ANOVA-based

13   method for calculating the repeatability of measurements from two years separately, and the GLMM-

14   based method to calculate overall adjusted repeatabilities for both years. We repeated the assessment for

15   the whole reflectance spectrum range and only the human-visible part, to assess the influence of the UV

16   component on the reliabilities of sampling methodologies. Our results reveal very high repeatability for lab

17   measurements and a lower, still moderate to high repeatability, for field measurements. Both increased

18   when limited to only the human-visible part, for all plumage patches except the throat, where we observed

19   the opposite trend. Repeatability between sampling methods was quite low including the whole spectrum,

20   but moderate including only the human-visible part. Our results suggest higher reliability for

21   measurements in the lab and higher power and accuracy of the GLMM-based method. They also suggest

22   UV reflectance differences amongst different plumage patches.

23   **Key words:** Adjusted repeatability, bird plumage, colourful displays, sexual selection,

24   spectrophotometry, tetrachromacy, ultraviolet.

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33   **Introduction**

34   Colour vision involves the capacity to discriminate amongst different wavelengths of light, independent

35   of their intensity [1,2]. Although colouration traits expressed in animals have proven essential components

36   to understand the nature of selection, sexual selection in particular, only relatively recently have scientists

37   appreciated the importance of a systematic understanding of both function and evolution of colouration, as

38   well as the mechanisms that underpin it [3]. Birds in particular, due to their colourful displays and the role

39   of their colour signals in fitness differentials, have traditionally been employed as prime model systems to

40   understand the causes and implications of colour evolution. However, the mechanisms of colour vision

41   and spectral information processing needed to understand how birds perceive colours remain areas with

42   more questions than answers.

43   Mate choice theory predicts that elaborately ornamented males can provide female birds with direct (if

44   ornamental traits reflect individual condition, useful individual attributes or somatic quality independent of

45   condition) and/or indirect fitness benefits ('good genes' or attractiveness for offspring – as conspicuous

46   and costly male traits indicate highly heritable viability) [4-6]. Therefore, birds with more elaborate colourful

47   displays are expected to enjoy a selective advantage given their higher mating chances [4,7].

48   Given the paramount significance of studies of bird plumage colouration in behavioural and evolutionary

49   ecology, methods for reliably and objectively quantifying such colouration are critical. Methods traditionally

50   used for colour assessment include colour ranks on an arbitrary scale [8], tristimulus colour models based

51   on human vision, such as   the Munsell system [9,10], reference colour swatches [11], or digital

52   photography [12-14]. However, although all these methods offer simple and affordable ways of colour

53   measurements in different analytical settings, they lack reliability and objectivity [15], as they are tuned to

54   the human visual system instead of the bird visual system.

55   Birds do not perceive colours in the same way as humans [16]. Birds have evolved a fourth cone type in

56   their eyes, with a pigment that is sensitive to ultraviolet light. And although we are still far from

57   understanding exactly how colours are perceived by birds [3], progress is being made towards

58   understanding how colour vision works in general and how spectral information is processed by birds and

59   other non-human animals [2,17,18]. Methods have been developed for comparing colour patterns as birds

60   see them, using known properties of bird eyes and generating detailed formal descriptions of colour

61   spaces and the equations used to plot them [19].

62   Since the 1990s, a wide range of further methods for analyzing spectrophotometry data have emerged

63   [3]. This development stems largely from the revival of interest in UV vision and tetrachromacy in birds

64   and the fact that birds can see colours that humans cannot experience [20,21]. This raises the possibility

3

65   of the existence of plumage patterns invisible to the human eye, and mate choice behaviours based on

66   the ultraviolet part of the bird reflectance spectrum have been discovered [22,23]. Therefore, reliable and

67   objective ways of quantifying bird coloration, not dependent on human vision, are at a premium. Miniature

68   diode-array spectroradiometer systems, lighter, more portable and affordable than previous spectrometry

69   systems but as precise and objective in colour quantification, have provided popular tools for colour

70   communication studies [3].

71   Two traditional ways of assessing bird plumage colouration with spectrophotometers have been

72   reported in the literature. Measurements may be taken directly on the bird, applying the pointer of the

73   spectrophotometer to plumage patches as they occur *in situ* [24-29]. Alternatively, measurements may be

74   taken in the lab, with feather samples collected from the field, applying the pointer (the cone-shaped piece

75   of black plastic on top of the probe used to direct the light from the source in a given angle) to "plumage

76   patches" created by mounting these feathers on a flat surface in a way that mimics the original plumage

77   structure [30-38]. Despite the popularity of the use of spectrophotometers for colour assessment and the

78   growing number of studies on bird colouration, few studies have rigorously assessed the consistency of

79   both methods for measuring the colouration of plumage patches, and the repeatability of results obtained

80   when using either one or the other (see [39] for a comparison of carotenoid-based plumage coloration in

81   great tits).

82   Additionally, there is little consensus on how to best quantify the reliability, or repeatability, of spectral

83   measurements. The most common measure of repeatability, or more precisely, the coefficient of intraclass

84   correlation (r_i), can be formally defined as the proportion of the total variance explained by differences

85   among groups [40,41]:

86

87    $$r_i = \sigma_\alpha^2 / (\sigma_\alpha^2 + \sigma_\varepsilon^2) \hspace{5cm} \text{(eqn 1)}$$

88

89   where σ_α^2 is the between-group variance and σ_ε^2 the within-group variance, whereas the sum of both

90   comprises the total phenotypic variance [41]. Until recently, the most common ways to estimate

91   repeatabilities from data with Gaussian errors have employed the correlation-based method [40] or the

92   ANOVA-based method, commonly used by behavioural and evolutionary ecologists [42,43]. However,

93   Nakagawa and Schielzeth [41] developed an innovative R-based function for calculating GLMM-based

94   repeatability estimates, which allows for confounding variables to be factored out and calculates the

95   confidence intervals for each repeatability calculation, inferred from distributions of repeatabilities obtained

96   by parametric bootstrapping.

4

97   Our aim is twofold. We first compare two different methods for measuring melanin-based plumage

98   ornamentation to determine which one allows to obtain more repeatable and reliable measures. The

99   methods consist in measuring the feather colouration either directly on the bird in the field or on feather

100 samples in the laboratory. We then compare two statistical methods to assess repeatability, one ANOVA-

101 based and another one GLMM-based, and determine the pros and cons of each of them. We hypothesize

102 that measuring feather colouration in the lab will yield more repeatable and reliable measures, as it avoids

103 the logistic, technical and animal welfare limitations imposed by the field method and provides more

104 controlled conditions during the measurements. Also, we predict more realistic and accurate repeatability

105 estimates with the GLMM-based statistical method, as it allows us to introduce more sources of variation

106 in each analysis. We use the European barn swallow *Hirundo rustica* as a model species. To the best of

107 our knowledge, this is the first time GLMM-based repeatability estimates have been used to assess the

108 reliability of melanin-based plumage colouration measurements.

109

110 **Methods**

111 **Field work and data collection**

112 Field work was carried out during May-August 2009 and March-August 2010 in multiple sites, mostly

113 farmlands, around the Falmouth area in Cornwall, UK. A total of 59 adult European barn swallows (21 in

114 2009 and 38 in 2010), were caught using mist nets, ringed, morphometric measurements taken, plumage

115 reflectance spectra quantified in the field and feather samples collected for subsequent assessment in the

116 lab.

117 Colour was quantified based on Endler and Mielke's [19] approach, using a USB2000

118 spectrophotometer (Ocean Optics, Dunedin, Florida), and a xenon flash lamp (Ocean Optics). Before

119 using the spectrophotometer, we calibrated it by setting the white and black references, i.e., we "told" the

120 machine which colour we want to be considered as the 100% reflectance (white) standard, and the 0%

121 reflectance (dark) standard, so that the rest of the colour measurements are determined in relation to

122 those maximum and minimum possible reflectance values. We used a WS-1 SS Diffuse Reflectance

123 Standard, a diffuse white plastic >98% reflective from 250-1500 nm, as the white reference (100%

124 reflectance), and a piece of black velvet as the dark standard (0% reflectance) to correct for the noise

125 when no light is reaching the sensor. At the far end of the reflection probe/light source, we put a non-

126 reflective black pointer cut in a 45 degree angle, to avoid mismeasurement derived from the white light

127 directly reflected by the plumage reaching the sensor [15]. Using the spectra acquisition software package

128 OOIBase (Ocean Optics), we measured the reflectance of four body regions, namely the throat, breast,

5

129   belly and vent of each bird. For the measurements of feather samples in the lab, we collected enough

130   feathers from live birds as to being able to mount them one on top of the other and simulate the original

131   pattern found on live birds. We mounted the feathers on a piece of black velvet to avoid background noise.

132   Once we had tested for the reliability of the measures obtained with both sampling methods separately,

133   we averaged the three measurements for each method and used these average values to test the

134   comparability between field and lab measurements.

135   We also used Endler and Mielke's [19] method to calculate brightness, chroma and hue, parameters

136   generally used to specify a colour. Using their equations and the mathematical software Matlab (The

137   MathWorks Inc., Natick, MA), we got the spectral sensitivity functions of the cones corrected for the cut

138   points of oil droplets, calculated the quantal catch for each photoreceptor and converted those quantal

139   catches into dimensional colour space coordinates in a tetrahedral colour space (Fig. 1). Chroma is

140   defined as the strength of the colour signal or the degree of difference in stimulation among the cones,

141   and it is proportional to the Euclidean distance from the origin, that is, the distance from the bird grey

142   (achromatic) point to each point, specified by three space coordinates. Perception of hue depends of

143   which cones are stimulated, and in tetrahedral colour space, it is defined by the angle that a point makes

144   with the origin. As bird colour space is 3D, hue is defined by two angles, analogous to latitude and

145   longitude in geography [19].

146   Brightness is defined as the summed mean reflectance across the entire spectral range ($R_{300-700}$;

147   [44,45]). As well as these parameters, we included UV chroma, a measure of spectral purity, into our

148   analysis, which was calculated as the proportion of reflectance in the UV part of the spectrum ($R_{300-400}$) in

149   relation to the total reflectance spectrum ($R_{300-700}$; [46]). Cone sensitivities and oil droplet cut points were

150   taken from Bowmaker et al. [16], Hart [47], Vorobyev et al. [48], Govardovskii et al. [49], and Hart and

151   Vorobyev [50].

152   Although all the avian families investigated show plumages reflecting significant amounts of UV light

153   (see [51] for a review), in the particular case of barn swallows, ventral plumage shows a noisy reflectance

154   pattern in the UV part of the spectrum and does not exhibit a clear ultraviolet reflectance peak (Fig. 2;

155   [34]). For this reason, we calculated the same colour variables both including and not including the UV

156   part of the reflectance spectrum, and carried out a repeatability assessment using either the whole

157   reflectance spectrum (300-700 nm) or only the human-visible part (400-700 nm). When using only the

158   visible part, we did not include UV chroma, for obvious reasons, nor hue, as values are identical in both

159   cases.

6

160   Due to the way data were collected, the three plumage colouration measurements taken in the field in

161   2009 covered a wider area of each plumage patch than the measurements made on feather samples,

162   which were restricted to the area covered by the bunch of feathers plucked from each patch on each

163   individual. In 2010, however, the three field measurements were taken approximately in the same

164   plumage area for each patch.

165

166   **ANOVA-based method**

167   In order to test for the reliability of both sampling procedures (described above) separately, we

168   calculated the repeatability for colour variables in the four patches for the different procedures according

169   to Lessells and Boag [43], Senar [52] and Quesada and Senar [39]. Repeatabilities were computed from

170   the mean squares of ANOVA on three repeated measures per individual. Both in field and lab procedures,

171   the second and third measurements were made after removing the reflection probe/light source and the

172   black pointer on top of it and placing it again on the colouration patch. IV took all the measurements.

173   Once we calculated the "within-method" repeatabilities, i.e., the repeatabilities for each sampling

174   method, we averaged the three measurements per patch per individual and assessed the "between-

175   method" repeatability, i.e. the repeatability of measurements across procedures, but this time the ANOVA

176   was carried out on two repeated measures per individual, one from the field and another one from the lab.

177   We repeated this process for both 2009 and 2010 data separately.

178

179   **GLMM-based method**

180   We used a modified version of the R function *R.Anson*, which is itself a modification of rpt.remlLMM

181   function [41]. We fitted two random-effect terms (individual identity and year) in our linear mixed-effects

182   models, and calculated the adjusted repeatability estimate as:

183

184    $r_i = \sigma_\alpha^2 / (\sigma_\alpha^2 + \sigma_\varepsilon^2 + \sigma_r^2)$ (eqn 2)

185

186   where σ_r^2 is the year variance.

187   In order to have a general idea of repeatability for each patch, we included all the colour variables in a

188   principal component analysis (PCA) and calculated the repeatability (and confidence intervals) for the first

189   component (PC1) within each plumage patch.

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191   As we conducted multiple statistical tests on data subsets that are not likely to be biologically

192   independent of each other (i.e. different components of the spectra, same metrics on different years, or

193   same metrics in the lab and in the field), there was an increased probability of type I error rates. To control

194   for this increased probability, we corrected our p-values for multiple testing based on the sequentially

195   rejective Bonferroni procedure of Holm [53], using the *p.adjust* function from the *stats* package in R [54].

196   All the statistical analyses were carried out using R [54,55].

197

198   **Ethics Statement**

199   AMcG and MRE had a Home licence which covered taking feather samples as well as other activities

200   (Home Office Project Licence Number 30/2740). MRE was the project licence holder.

201   All work was carried out on private residencies and farms with the express permission of the landowners

202   in question. Contact details of the landowners can be provided by the authors upon request and after

203   asking the landowners for permission, in order to respect their privacy.

204   Our field study did not involve any endangered or protected species. The specific locations of the study

205   are provided as supporting material.

206   Birds were caught using mist nets under licence (AMcG BTO A licence Holder No.4947).

207

208   **Results**

209   **ANOVA analyses**

210   In 2009, when including the whole spectrum in the analyses, measuring plumage colouration in the lab

211   proved to be a reliable method. Brightness, UV chroma, chroma and hue latitude and longitude being

212   highly repeatable for all the patches, and hue latitude in the throat being less repeatable (r_i=0.418,

213    $F_{21,44}$=3.157, P=0.01; Table 1). The method of measuring the plumage colouration in the field (in three

214   different points, covering a wider area of each patch) was also quite consistent but with overall lower

215   values of repeatability, although still reasonably high, for all the variables and patches, being especially

216   low for hue latitude in breast (r_i=0.382, $F_{20,42}$=2.856, P=0.025) and vent (r_i=0.394, $F_{21,44}$=2.955, P=0.017;

217   Table 1).

218   When including only the visible part of the spectrum in the analysis, overall repeatability declined. The

219   lab method again proved to be the most reliable, with high values of repeatability for brightness and

220   chroma in all the patches. The field procedure was moderately repeatable for belly and throat, but showed

221   low repeatability for brightness in the breast (r_i=0.36, $F_{20,42}$=2.6885, P=0.038) and for chroma in the vent

222   (r_i=0.428, $F_{21,44}$=3.241, P=0.007; Table 2).

223   The values of repeatability across the field and laboratory procedures were very low for all the patches

224   measured, both considering the whole reflectance spectrum or only the visible part ($r_i<0.35$ and $P>0.05$ in

225   all cases), suggesting a lack of consistency across the two assessment methods for melanin-based

226   plumage colouration. Repeatabilities of brightness measurements were slightly higher for the whole

227   spectrum than for only the visible part, in all the patches except from the vent. However, in this case,

228   including only the visible part of the spectrum yielded much more repeatable chroma values than including

229   the whole range, sometimes even turning negative repeatability values into positive, e.g. for the belly

230   (whole range: $r_i=-0.507$, $F_{20,21}=0.327$, $P=1$; only visible range: $r_i=0.346$, $F_{20,21}=2.056$, $P=0.599$), or the vent

231   (whole range: $r_i=-0.758$, $F_{21,22}=0.138$, $P=1$; only visible range: $r_i=0.296$, $F_{21,22}=1.842$, $P=0.657$; Table 1 and

232   Table 2).

233

234   In 2010, when including the whole spectrum in the analyses, repeatability measurements in the field

235   (taken approximately in the same point within each patch) yielded considerably higher results than in

236   2009, with all the r_i values above 0.60, except from hue latitude in the throat ($r_i=0.515$, $F_{37,75}=4.186$,

237    $P<0.0001$), and with most of the values ranging from 0.74 to 0.91, except for brightness in the breast

238   ($r_i=0.611$, $F_{37,76}=5.710$, $P<0.0001$), hue latitude in the belly ($r_i=0.63$, $F_{37,76}=6.1$, $P<0.0001$), hue latitude in

239   the vent ($r_i=0.629$, $F_{37,76}=6.088$, $P<0.0001$) and hue longitude in the vent ($r_i=0.679$, $F_{37,76}=7.356$,

240    $P<0.0001$). In the lab, repeatability values ranged from 0.71 to 0.95 in most of the patches, except from

241   hue latitude in the throat ($r_i=0.65$, $F_{37,76}=6.569$, $P<0.0001$), and repeatability was overall higher than when

242   measuring it on live birds, except from UV chroma in the belly ($r_i=0.857$, $F_{37,76}=18.986$, $P<0.0001$), breast

243   ($r_i=0.788$, $F_{37,76}=12.117$, $P<0.0001$) and vent ($r_i=0.819$, $F_{37,76}=14.571$, $P<0.0001$) and hue latitude in the

244   breast ($r_i=0.722$, $F_{37,76}=8.808$, $P<0.0001$), where it was slightly lower. Repeatability values were similar to

245   the ones obtained in 2009 (Table 3).

246   When doing the analysis including only the visible part of the spectrum, measuring colouration in the lab

247   was again the most reliable method of both, with all the r_i values above 0.91, except for chroma in the

248   throat ($r_i=0.881$, $F_{37,76}=22.964$, $P<0.0001$). Field procedure still yielded high repeatability values, with

249   brightness in the breast ($r_i=0.569$, $F_{37,76}=4.959$, $P<0.0001$) and chroma in the vent ($r_i=0.696$, $F_{37,76}=7.839$,

250    $P<0.0001$) being the only measurements with values below 0.81. For both methods, repeatability values

251   were higher than in 2009 for all the variables within all the plumage patches (Table 4).

252   Repeatabilities across field and lab methods in 2010 were quite heterogeneous including the whole

253   spectrum in the analyses: moderately high for hue longitude in the belly ($r_i=0.794$, $F_{37,38}=8.732$, $P<0.0001$)

254   and breast ($r_i=0.657$, $F_{37,38}=4.818$, $P<0.0001$), moderate for vent hue latitude ($r_i=0.463$, $F_{37,38}=2.723$,

9

255   P=0.018) and longitude (r_i=0.561, $F_{37,38}$=3.553, P=0.001), belly hue latitude (r_i=0.431, $F_{37,38}$=2.515,

256   P=0.034) and breast brightness (r_i=0.482, $F_{37,38}$=2.861, P=0.012), and low for breast chroma (r_i=0.326,

257     $F_{37,38}$=1.966, P=0.21), throat UV chroma (r_i=0.321, $F_{37,38}$=1.944, P=0.312) and chroma (r_i=0.274,

258     $F_{37,38}$=1.755, P=0.576) and vent brightness (r_i=0.349, $F_{37,38}$=2.070, P=0.141). For the rest of the cases,

259   repeatabilities were very low (r_i<0.23 and P>0.05 for all the cases). When including only the visible part of

260   the spectrum, repeatability was moderate to high and had a significant effect for both brightness and

261   chroma in the breast, and for chroma in the vent and in the belly, whereas it was quite low and non-

262   significant for brightness in the belly (r_i=0.217, $F_{37,38}$=1.556, P=0.899), and very low for both variables in

263   the throat. Repeatability values for chroma in all the patches except for the throat were much higher than

264   when we included the whole spectrum range, e.g. in the belly (whole range: r_i=-0.049., $F_{37,38}$=0.906, P=1;

265   only visible range: r_i=0.75, $F_{37,38}$=7.005, P<0.0001) and in the vent (whole range: r_i=0.224, $F_{37,38}$=1.579,

266   P=0.657; only visible range: r_i=0.416, $F_{37,38}$=2.428, P=0.047; Table 3 and Table 4).

267

268   **Generalized Linear Mixed Model analyses**

269   When we included the whole spectrum in the analyses, for all the principal component analyses carried

270   out within each patch for field and lab measurements, PC1 accounted for more than a 53% of the total

271   variance, except for vent measurements in the field (where it explained a 49% of the total variance) and

272   for measurements in the throat (where it explained between 44% and 47%). When including only the

273   visible part of the spectrum, PC1 explained a 67% of the total variance for vent measurements in the field,

274   and between 75% and 93% in the rest of the cases.

275   Repeatability of feather colour measurements was much higher and confidence intervals smaller when

276   quantifying colouration from measurements taken in the lab than when doing it on measurements taken on

277   live birds in the field, both including the whole spectrum in the analyses or only the visible part, being

278   particularly high in the breast (whole range: r_i=0.916, 95%CI=0.855, 0.943, P<0.0001; visible range:

279   r_i=0.927, 95%CI=0.872, 0.95, P<0.0001). All the repeatability values from lab measurements ranged

280   between 0.71 and 0.93 and were highly significant (P<0.0001).

281   When using field measurements, repeatabilities were still moderately high (all r_i values above 0.50) and

282   higher when including only the visible spectrum range in the analyses than including the whole range,

283   except in the throat (whole range: r_i=0.629, 95%CI=0.303, 0.874, P<0.0001; visible range: r_i=0.564,

284   95%CI=0.266, 0.816, P<0.0001; Fig. 3).

285   Repeatabilities across both field and lab methods yielded higher results when we included only the

286   visible spectrum in the analyses than when we included the whole spectrum. Leaving the values for throat

10

287   apart, as here repeatability was not significantly different from zero no matter the spectrum range we

288   included in the analyses, r_i ranged between 0.19 and 0.41 when including the whole spectrum, and

289   between 0.44 and 0.62 when including only the visible part (Fig. 3). All the repeatabilities in belly, breast

290   and vent were significant except for the belly when including the whole spectrum (r_i=0.189, 95%CI=0,

291   0.415, P=0.069), but it became significant and higher when only the visible spectrum was included in the

292   analyses (r_i=0.503, 95%CI=0.281, 0.667, P<0.0001, Fig. 3).

293

294   **Discussion**

295   **Measuring plumage ornamentation to gain repeatable and reliable measures**

296   Measuring plumage colouration from feather samples in the lab proved to be a highly reliable method,

297   with high values of repeatability in general for all the variables and patches in 2009, 2010 and when

298   applying the GLMM-based method for both years. Measurements taken directly on specimens in the field

299   showed a reasonable extent of reliability, but with overall lower values of repeatability compared to the lab

300   for most of the variables measured on different patches. As an exception, some variables in the throat in

301   2009 and UV chroma measurements in the belly, breast and vent in 2010 when considering the whole

302   spectrum, yielded higher values of repeatability when measured in the field, only when applying the

303   ANOVA-based method.

304   A potential explanation for our findings is that the throat patch is smaller and much darker than the rest

305   of the patches. The feathers of the throat patch are also considerably smaller. Therefore, it is often quite

306   difficult to obtain a reliable reflectance measurement with such a limited amount of photons reaching the

307   spectrophotometer probe. Also, it is more difficult to create a "plumage patch" in the lab with a feather

308   arrangement similar to the bird's original one and big enough to be able to apply the spectrophotometer

309   pointer to it. An alternative explanation is that the UV part of the spectrum shows a highly noisy pattern,

310   thus highly consistent UV chroma repeatabilities across field or lab measurements may not necessarily be

311   expected. This latter alternative may explain why repeatability values in the lab for UV chroma

312   measurements were higher in the field in 2010, whereas the rest of the repeatability values consistently

313   tended to be higher in the lab.

314   When applying the GLMM-based method, repeatability was moderate to high within all the patches for

315   field measurements, whereas it was considerably higher, and the confidence intervals considerably

316   narrower, for lab measurements. This finding suggests that lab-based measures are more reliable ways of

317   assessing melanin-based colouration.

11

318   When comparing both methods, the values obtained in 2009 for different variables measured in different

319   plumage patches directly on field birds were poorly repeatable compared to the values obtained for the

320   same variables measured from feather samples in the lab, and non-significant in all cases. In 2010, in

321   contrast, repeatabilities were higher and significant for certain metrics in certain patches only. These

322   results stand in marked contrast to the positive results of another study, which compared the

323   repeatabilities between both colouration assessment procedures for carotenoid-based plumage [39].

324   There can be several reasons for this difference: for example, due to the different characteristics of the

325   two types of pigments, carotenoid-derived colouration is more variable among individuals than melanin-

326   based colouration [56], and repeatability of a character increases with variability [52]. In order to increase

327   the repeatability of some measurements, a possible solution could be to increase the number of

328   measurements, for example from three to five, as it has already been done by several authors [22,28,32].

329   Unfortunately, this is not an option when working with live birds in the field, as we would be increasing the

330   manipulation times and, consequently, the stress levels to an unacceptable degree, although it can be

331   applied when assessing colouration in the lab on feather samples [39].

332   As mentioned above, the three plumage colouration measurements taken in the field in 2009 covered a

333   wider area of each plumage patch than in 2010, when the three field measurements were taken

334   approximately in the same plumage area for each patch, being this area also the one from which the

335   majority of feathers for lab measurements were taken. Due to the different ways in which data were

336   collected in the field each year, repeatability of 2009 field measurements can be taken as an estimate of

337   colouration consistency within the plumage patches. Our results suggest a moderate to high within-patch

338   consistency for melanin-based ventral colouration in our model species. The comparability of both

339   procedures in 2009 may have been compromised, although the repeatability of the 2010 samples was

340   higher even for lab measurements, especially when considering only the visible part of the spectrum. This

341   may be indicative of higher patch colouration homogeneity in 2010. Also, the higher lab repeatabilities in

342   2009 than the field ones could be argued to be a function of field measures being taken over bigger

343   plumage areas. But the fact that we still get higher lab than field measurements in 2010 suggests practical

344   limitations to repeatability measurements in the field.

345   Consequently, collecting feathers from birds and assessing their colouration in the lab, as well as being

346   more convenient, minimising risk to a sensitive device like a spectrophotometer and reducing handling

347   times of the animals [39], is a more reliable method for assessing melanin plumage colouration than

348   doing so directly on live birds, according to our results.

349

350   **Assessing repeatability**

351   The GLMM-based method [41], applied to data from both years, allowed us to control for year effects by

352   adding the year variance into the total variance calculation, so that we could obtain the adjusted

353   repeatability for data from both years. The PCA allowed to create composite variables accounting for

354   almost 50% of the total variance in the metrics taken from each patch, which therefore, made it possible to

355   estimate the overall repeatability within each patch for both methods separately and across methods.

356   The possibility of calculating adjusted repeatabilities by including year as a random factor, together with

357   the reduction in the number of variables accounting for a great proportion of the total variance achieved by

358   the PCA, considerably reduced the amount of multiple tests necessary for repeatability calculation. Thus,

359   the p-values obtained with this method were less affected by Bonferroni corrections than those obtained

360   with the ANOVA-based method, reducing the probability of type II errors and increasing the power of this

361   repeatability-calculation method.

362   Additionally, this reduction in the number of variables offered us the possibility of getting a general view

363   of the colour measurement repeatability within each patch. As our variables are all derived from the

364   reflectance spectra obtained on each colouration patch through the spectrophotometer, comparing the

365   repeatability of all the different variables within the patches, as well as increasing the probability of type II

366   errors, can be redundant. The PCA-GLMM combination, in contrast, made it possible to compare the first

367   principal components within each patch, which can be regarded as whole spectrum estimates, as they

368   gather a great proportion of the total variance contained in all the variables originally extracted from the

369   reflectance spectra measurements. And it also allowed us to do so with data from both years.

370   Finally, thanks to the use of the GLMM-based method, we could calculate confidence intervals, useful

371   indicators of the reliability of our repeatability estimates, additionally to just p-values. That way, and

372   together with the advantages mentioned above, it was possible to get a more complete and reliable overall

373   perspective of the question being studied.

374   The fact that almost all the repeatability measurements, and especially the repeatabilities across field

375   and lab methods (in patches other than the throat), were higher when including only the human-visible

376   spectrum in the analyses , suggests that the noisy reflectance pattern in the UV part of the spectrum may

377   be decreasing the repeatability and underestimating the comparability of the two methods. For throat

378   plumage, however, we observed the opposite trend, with higher repeatability values when including the

379   whole spectrum, which could be indicative that the UV part of the spectrum is more important in the throat

380   than in the rest of the patches. We find support for this idea when looking at reflectance spectra plots for

381   different patches (Fig. 2): throat reflectance spectra, although showing also quite a noisy pattern for the

13

382   UV part, and unlike the rest of the patches' spectra, tends to show UV reflectance peaks in both sexes.

383   Further work is needed to find out whether there are UV reflectance differences amongst different

384   plumage patches.

385

386   **Conclusions**

387   Our results suggest that collecting feathers from live animals and assessing colouration in the lab is a

388   better approach for measuring plumage ornamentation in order to gain repeatable and reliable results

389   compared to direct measures on live birds in the field. In addition, since it is easier on equipment and

390   minimises the length of time birds need to be handled (minimising the stress levels inflicted on them),

391   feather sampling would appear to be the best method available.

392   In addition, from a statistical point of view, our results support the superiority of the GLMM-based method

393   [41] for repeatability calculation, as it enables random factors to be accounted for and can calculate

394   adjusted repeatability values, which are more accurate than those calculated using other (e.g., ANOVA)

395   methods and increase the power of the tests. The reduction in the number of variables gives us a general,

396   patch by patch overview of the problem being studied, and the confidence intervals allow us to test the

397   reliability of our own repeatability estimates.

398   Finally, we have also shown that it is important to check for the effect that the UV part of the spectrum

399   could be exerting on repeatability calculations, as the capability of the plumage to reflect the UV light could

400   have different biological implications in different plumage patches.

401

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Table 1: ANOVA-derived Repeatabilities in 2009 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (UV+Visible spectrum).

	UV+Visible range							
	Belly		Breast		Throat		Vent	
	F	r_i	*F*	r_i	*F*	r_i	*F*	r_i
Repeatability field								
Brightness	6.0446	**0.627***	3.1183	**0.414***	12.273	**0.79***	6.1868	**0.634***
UV Chroma	10.203	**0.754***	14.171	**0.814***	13.861	**0.811***	5.3196	**0.59***
Chroma	9.0161	**0.728***	11.238	**0.773***	10.714	**0.764***	4.5611	**0.543***
Hue latitude	4.0921	**0.508***	2.8564	**0.382***	4.4146	**0.532***	2.9545	**0.394***
Hue longitude	13.025	**0.8***	9.1422	**0.731***	24.348	**0.886***	4.6771	**0.55***
Repeatability lab								
Brightness	13.188	**0.802***	14.777	**0.821***	8.2092	**0.706***	20.212	**0.865***
UV Chroma	46.493	**0.938***	23.015	**0.88***	34.895	**0.919***	25.561	**0.892***
Chroma	42.489	**0.932***	26.975	**0.896***	62.481	**0.954***	29.052	**0.903***
Hue latitude	11.986	**0.785***	6.5101	**0.647***	3.1573	**0.418****	23.024	**0.88***
Hue longitude	25.986	**0.893***	9.2833	**0.734***	6.0119	**0.625***	27.291	**0.898***
Comparison field-lab								
Brightness	1.4636	**0.188**	2.0702	**0.349**	0.865	**-0.072**	1.8043	**0.287**
UV Chroma	1.3153	**0.136**	1.3591	**0.152**	0.7499	**-0.143**	0.9006	**-0.052**
Chroma	0.327	**-0.507**	1.3715	**0.157**	1.0143	**0.007**	0.1376	**-0.758**
Hue latitude	0.9084	**-0.048**	0.8635	**-0.073**	1.2523	**0.112**	0.706	**-0.172**
Hue longitude	1.7364	**0.269**	1.7513	**0.273**	0.6709	**-0.197**	1.1055	**0.05**

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$

20

Table 2: ANOVA-derived Repeatabilities in 2009 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (Only Visible part of the spectrum).

	Visible range							
	Belly		Breast		Throat		Vent	
	F	r_i	F	r_i	F	r_i	F	r_i
Repeatability field								
Brightness	4.8958	**0.565***	2.6885	**0.36***	11.221	**0.773***	5.6072	**0.606***
Chroma	6.7724	**0.658***	6.4868	**0.646***	6.6207	**0.652***	3.2416	**0.428**
Repeatability lab								
Brightness	11.818	**0.783***	15.892	**0.832***	6.9936	**0.666***	19.326	**0.859***
Chroma	28.865	**0.903***	21.117	**0.821***	29.402	**0.873***	23.838	**0.901***
Comparison field-lab								
Brightness	1.461	**0.187**	2.0097	**0.335**	0.6804	**-0.19**	1.8902	**0.308**
Chroma	2.0563	**0.346**	1.6683	**0.25**	1.1396	**0.065**	1.8415	**0.296**

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$.

21

Table 3: ANOVA-derived repeatabilities in 2010 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (UV+Visible spectrum).

	UV+Visible range							
	Belly		Breast		Throat		Vent	
	F	r_i	F	r_i	F	r_i	F	r_i
Repeatability field								
Brightness	16.422	**0.837***	5.7104	**0.611***	17.398	**0.846***	14.534	**0.819***
UV Chroma	25.333	**0.89***	15.853	**0.832***	12.357	**0.791***	16.829	**0.841***
Chroma	24.037	**0.885***	14.749	**0.821***	22.212	**0.876***	12.377	**0.792***
Hue latitude	6.0997	**0.630***	9.7411	**0.744***	4.1857	**0.515***	6.0878	**0.629***
Hue longitude	23.669	**0.883***	31.363	**0.910***	9.8914	**0.748***	7.3556	**0.679***
Repeatability lab								
Brightness	55.197	**0.947***	31.387	**0.910***	30.9	**0.909***	44.036	**0.934***
UV Chroma	18.986	**0.857***	12.117	**0.788***	17.544	**0.847***	14.571	**0.819***
Chroma	25.375	**0.890***	25.936	**0.893***	27.679	**0.899***	39.854	**0.928***
Hue latitude	8.3908	**0.711***	8.8076	**0.722***	6.5692	**0.650***	9.1422	**0.731***
Hue longitude	37.357	**0.924***	31.683	**0.911***	11.664	**0.781***	37.517	**0.924***
Comparison field-lab								
Brightness	1.3041	**0.131948**	2.8612	**0.482***	0.7642	**-0.134**	2.0703	**0.349**
UV Chroma	0.5572	**-0.284**	1.059	**0.029**	1.9441	**0.321**	1.5873	**0.227**
Chroma	0.9059	**-0.049**	1.9662	**0.326**	1.755	**0.274**	1.5788	**0.224**
Hue latitude	2.5152	**0.431***	1.2479	**0.110243**	0.7839	**-0.121**	2.7232	**0.463***
Hue longitude	8.7322	**0.794***	4.8175	**0.657***	**0.9731**	**-0.014**	3.5525	**0.561***

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$

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Table 4: ANOVA-derived Repeatabilities in 2010 plumage colouration measurements taken from live birds in the field, feather samples in the lab, and across both procedures (Only Visible part of the spectrum).

	Visible range							
	Belly		Breast		Throat		Vent	
	F	r_i	F	r_i	F	r_i	F	r_i
Repeatability field								
Brightness	14.408	**0.817***	4.959	**0.569***	16.59	**0.839***	13.527	**0.806***
Chroma	22.566	**0.878***	22.163	**0.876***	20.007	**0.864***	7.8386	**0.696***
Repeatability lab								
Brightness	59.713	**0.951***	34.778	**0.918***	34.133	**0.917***	49.55	**0.942***
Chroma	51.455	**0.944***	57.897	**0.950***	22.964	**0.881***	72.314	**0.959***
Comparison field-lab								
Brightness	1.5559	**0.217**	3.1819	**0.522****	0.679	**-0.191**	2.3696	**0.406.**
Chroma	7.0047	**0.750***	7.0261	**0.751***	1.0876	**0.042**	2.4275	**0.416***

'***' $P<0.001$; '**' $P<0.01$; '*' $P<0.05$ '.' $P<0.1$

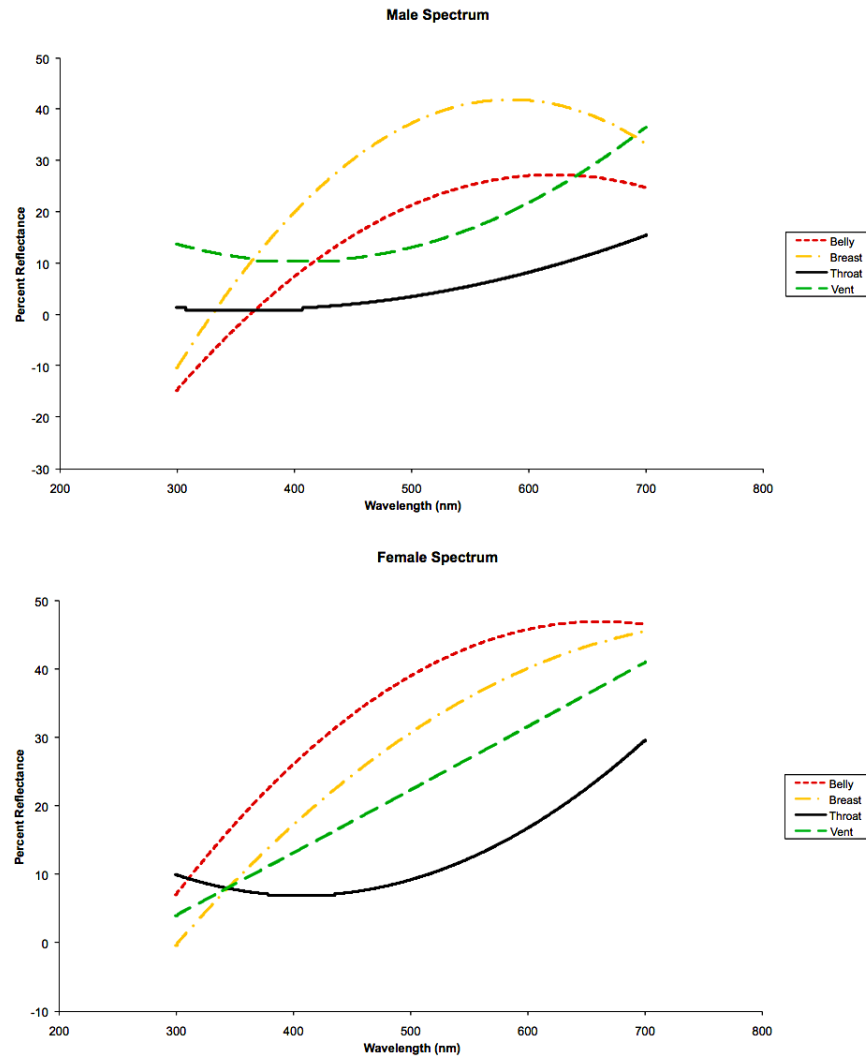**Figure 1**: The avian tetrahedral colour space (from Endler and Mielke, 2005).

Figure 2: Reflectance spectra for belly, breast, throat and vent patches of male and female barn swallow (regression lines).
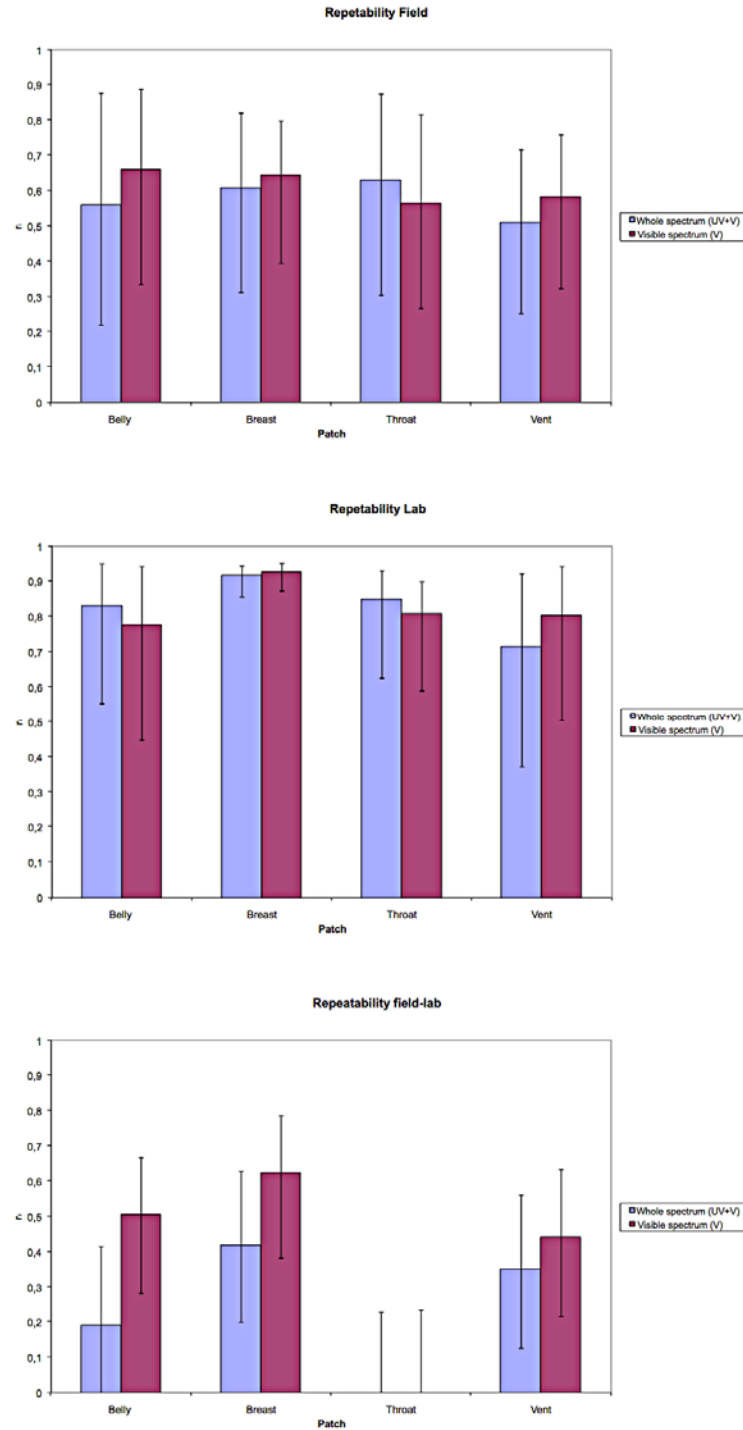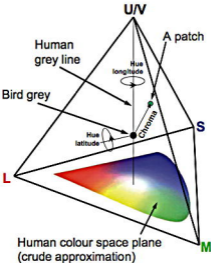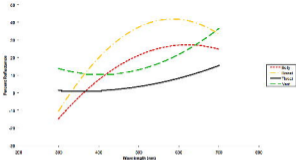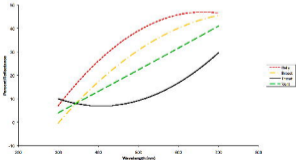
Figure 3: GLMM-derived repeatabilities (± 95% CI) in 2009-2010 plumage colouration measurements taken a) from live birds in the field, b) from feather samples in the lab, and c) across both procedures, both when including the whole light spectrum or only the human-visible spectrum in the analyses.
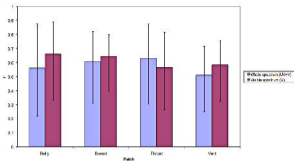
26

U/V

A patch

Human grey line

Hue longitude

Bird grey

Hue latitude

Chroma

S

L

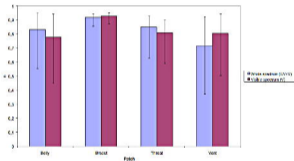Human colour space plane (crude approximation)

M

Male Spectrum

Female Spectrum

Repeatability Field

Repeatability Lab

Repeatability field/lab